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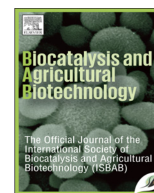
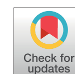


Template synthesized polypyrroles as a carrier for diastase alpha amylase immobilization



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ABSTRACT

The ability of Polypyrroles in assimilating enzymes promises new candidates for enzyme immobilization. The surfactants SDS, CTAB and TWEEN -80 have been employed as templates in order to prepare spherical polypyrrole particles via micro-emulsion polymerization method. The diastase alpha amylase extracted from malt is the model enzyme for our studies. Both free and immobilized enzymes were characterized using IR, UV, BET and SEM analysis. The biochemical characterization includes optimization of pH, temperature, reusability and storage stability. The kinetic parameters K_m and V_{max}were also evaluated using Lineweaver- Burk plots. The K_m values were found to be increasing and V_{max} values decreases for all immobilized enzymes. After 15 cycles of reuse, immobilized enzymes retained almost 50% of its initial activity. The thermal stability and storage stability exhibited by immobilized enzymes ensure the contribution of supports for future biochemical applications.

1. Introduction

Polypyrrole is one among the most fascinating conducting polymer because of its excellent characteristics like good chemical/thermal stability, high conductivity and ease of preparation in a range of solvents. This has led to wide potential applications of polypyrrole in various fields such as sensors, actuators, etc [Otero et al., 2004; Saxena and Malhotra, 2003]. Polypyrroles have been frequently studied as conducting polymer for its interaction with biological entities which inturn provided new candidates for enzyme immobilization. Moreover cotton fabrics incorporated with these polymers exhibited enhanced antimicrobial and antistatic properties and thereby served as excellent biocompatible polymer for industrial applications [Wang et al., 2004; Seshadri and Bhat, 2005].

In order to prepare polypyrroles with different morphology, polymerization of pyrrole in different surfactant systems have been developed in recent years. The variation in concentration of pyrrole monomer and surfactant has been proved to play a major role in tailoring the morphology of the polypyrrole (Abdolmaleki, A. Y. and Eisazadeh, H., 2012.). This is because surfactants induced pyrrole to grow in a restrained manner and resulted in polypyrrole (PPY) with a morphology having definite pattern. The polymer thus formed will show properties superior to those from a conventional aqueous solution [Berdichevsky and Lo, 2006; Huang et al., 2004].

Inorder to prepare polymer nanostructures, micro-emulsion polymerization had been developed. In this technique, particles were brought into the surfactant template so as to transform into spherical aggregates. The fabrication of micro-reactor vessel was done by the surfactant via its micelle formation, and the monomer was confined in a localized environment generated from encapsulation by the surfactant [Stejskal et al., 2003]. Compared with the aqueous solution and conventional emulsion polymerization, the micro-emulsion polymerization of PPY increased the extent of the pi-conjugation along the polymer backbone, and brought about the ordered arrangement of the macromolecular chains [Reung-U-Rai, A. et al., 2008].

Zhang et al. reported the controllable synthesis of polypyrrole nanostructures with different kinds of surfactants, including CTAB, dodecyltrimethylammonium (DTAB), octyltrimethylammonium, poly (ethylene glycol)mono-p-nonylphenyl ether (Opi-10), and sodium dodecyl sulfate (SDS) [Zhang et al., 2006]. Grady et al. reported the formation of nanostructured polypyrrole with controlled morphologies on atomically flat surfaces with adsorbed surfactant molecules as templates [Carswell et al., 2003]. Omastova' et al. conducted the synthesis of polypyrrole in the presence of anionic, cationic, and non-ionic types of surfactants [Omastova et al., 2003]. Kwon et al. reported that polypyrrole prepared without surfactant showed an arbitrary shape whereas, the polypyrrole samples prepared in presence of surfactant showed a spherical shape [Kwon et al., 2008]. From all these studies, it

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can be concluded that the surfactant provided a space to control morphology as template does.

Immobilization of an enzyme yields more stable robust biocatalyst which has high reusability, thermal and storage stability [Vinu et al., 2004, Mateo et al., 2007]. It thus paved way for decreasing the production costs and hence serves a vital part for many industrial and analytical applications. The so formed template synthesized polypyrrole is also amenable for variety of modifications which makes it an attractive substrate for neural scaffolds, sensors and other biomedical applications [Jugović et al., 2016]. The present work aims to utilize polypyrrole prepared in presence of surfactants as a support for diastase alpha amylase immobilization. The study also emphasized the role of surfactants in tailoring the structure of polypyrrole formed and focused on an ample scope for its ability in surface modification.

2. Materials and methods

2.1. Materials

Diastase alpha-amylase (Himedia Laboratories Pvt Ltd, Mumbai. Soluble Starch (potato) (S.D. Fine-Chem. Ltd, Mumbai), Albumin Bovine and Folin & Ciocalteu's Phenol Reagent (Sisco Research Laboratories Pvt. Ltd. Mumbai), NaOH (Universal Laboratories), and acetone (s.d fiNE Chem. Ltd), $CHCl_3$ (Spectrochem.) were used as received. Pyrrole (s.d fiNE CHEM. Ltd) was distilled prior to use. All chemicals used in the study were of analytical grade.

2.2. Preparation of polypyrrole in the presence of surfactants.

The anhydrous $FeCl_3$ (0.1 mol, 16.2 g) was dissolved in 100 ml distilled water in a reactor vessel containing magnetic stirring bar. To this solution, each surfactant with concentration above their cmc (cmc values are SDS- 8.2mM, CTAB- 1mM, and Tween 80–0.015mM) dissolved in 100 ml distilled water taken in another reactor vessel was added and mixed. The whole mixture was then stirred for 30 min until surfactant completely dissolved. The freshly distilled pyrrole (0.15 mol, 10.4ml) was first dispersed in 50 ml of distilled water and then inserted dropwise into the stirred mixture of the oxidant and surfactant. Immediate formation of black polypyrrole was clearly observed soon after the addition of the monomer. The polymerization was carried out for 24 h at room temperature with moderate stirring. Finally the polypyrrole was washed with acetone and dried in oven at 60^0C for 2 h.

The polypyrrole prepared in presence of anionic surfactant SDS (designated as PS) was treated with an excess of 1M NaOH for 12 h, filtered , washed with distilled water and dried at 60^0C in the oven. The anionic surfactant was thus removed leaving behind pure polypyrrole with controlled morphology. The cationic and non-ionic surfactants (which will be designated as PC and PT respectively) were removed by washing with methanol [Omastova et al., 2003]. The amphiphilic macromolecular chains of surfactants self-assembled into sphere-like micelles without any interference from the ionic oxidizing agent. Pyrrole monomers were encapsulated into more or less uniform spherical micelles driven by the hydrophobic interaction as shown in the Scheme 1. [Shaolin ,1994.; Soares et al., 2012].

2.3. Immobilization of alpha-amylase onto polypyrrole

The polypyrrole as support for the enzyme immobilization process was subsequently coupled to diastase alpha amylase. A definite concentration of the diastase alpha amylase was dissolved in different pH and a part of it was mixed with 1g supports. The enzyme solution was tested for its total protein concentration before and after immobilization using Folin & Ciocalteu's Phenol reagent and the colour developed was measured using shimadzu 160A UV-Visible spectrophotometer at 750nm [Lowry et al., 1951]. The enzyme immobilized support was then filtered and washed with respective buffer three times to remove unimmobilized enzymes from the support so that leaching in between the reaction conditions can be minimized. The supernatants collected were also analyzed for protein determination. The activities of free and immobilized enzymes were checked by its reaction with 1% starch solution. About 0.25g of the immobilized sample was then weighed and incubated with 2 ml of the starch solution at 40°C (to be optimized) for 10 min. The preparation was then centrifuged at 3,500 rpm for 5 min. The supernatant of the preparation was tested for its activity by arresting the reaction with 0.3 ml of 1M HCl. From this mixture- 0.5mL of aliquot was taken and to this solution 0.1 mL iodine solution was added and the blue colour developed was diluted with distilled water to a fixed volume. In the case of free enzyme activity assay, 0.1 ml of free enzyme solution was taken in a test tube to which 0.5 mL corresponding buffer and 1 ml starch was added and incubated at 50°C for 1 min. The absorbance was measured at 650 nm using UV spectrophotometric method. All the tests were performed in triplicate and the relative standard deviations were found to be less than 1%. The amount of enzyme which hydrolyzes 1 mg of starch in 1 min at optimum temperature and pH was considered as one unit of alpha-amylase activity.

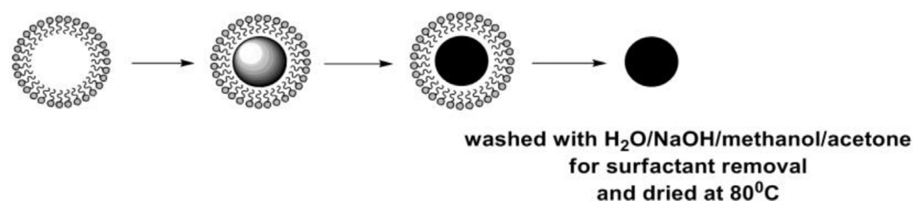
The enzyme coupled preparation was re-suspended in buffer solution at 4 °C for further analysis like optimization of concentration, time, temperature, protein loading, activity, storage stability, thermal stability and reusability. The kinetic parameters were also analyzed so as to find out Km and Vmax. The difference between the total protein concentrations before and after the enzyme coupling incubation step was used to find out the amount of enzyme that had been immobilized onto the carrier particles. The difference in protein concentration of the supernatants before and after immobilization gives the immobilization yield (IY). The ratio of activity of immobilized enzyme to the activity of the initial enzyme used in the immobilization reaction gives the activity yield. The ratio of activity yield to the immobilization yield gives the immobilization efficiency.

2.4. Characterization of immobilized enzymes

SEM images were obtained using the JEOL Model JSM –6390LV scanning electron microscope. The N_2 adsorption-desorption isotherms were obtained on a Micromeritics TriStar 3000 at 77 K under continuous adsorption condition. The total specific surface area was determined using the BET analyses. FT-IR spectrometry was done with a Thermo Nicolet, Avatar 370 FT-IR Spectrometer.

2.5. Optimization of immobilization parameters

The optimum pH at which maximum activity exhibited by both free and immobilized enzyme was assayed over a pH range 4-8 at 40°C. The



Scheme 1. Preparation of polypyrrole through surfactant template.

optimum temperature at which free and immobilized enzymes were able to preserve their native three dimensional structures without much loss in their activity was interpreted by incubating the enzyme reaction mixture at different temperatures from 30-60°C. Thermal stability of the enzyme was determined by pre-incubating free and immobilized enzyme at 30-100°C for 60 min, and assaying their residual activity. Thermal inactivation of enzymes with respect to time was obtained by pre-incubating both free and immobilized enzyme in respective buffer of optimum pH and maintaining the reaction mixture at its optimum temperature. After definite time interval, known amount of enzyme was withdrawn and tested for activity.

2.6. Determination of kinetic parameters

The kinetic parameters, Michaelis constant (K_m) and maximum rate (V_{max}) were determined from Lineweaver-Burk plot. Initial reaction rate was measured under optimum conditions of pH and temperature by varying the starch concentration and by measuring the initial rates of the reaction of alpha-amylase.

2.7. Reusability and storage stability

In the reusability studies, the activity was measured as per the method described in section 2.3. The immobilized enzyme was then filtered and washed several times with the buffer solution and fresh substrate was added to it for the next cycle of activity determination. The reaction was carried out at specific intervals continuously for 15 cycles. The storage stability of immobilized enzyme was analyzed by measuring their activity at fixed intervals from the immobilized enzyme stored in buffer media at 4°C for 30 days.

3. Results and discussions

3.1. FT-IR spectra of polypyrrole prepared in presence of surfactants

The FTIR spectra of PPY prepared in presence of different types of surfactants are shown in Fig. 1a. The characteristic peaks of polypyrrole was clearly observed. For instance, the peak at about 3436 cm^{-1}, 3434 cm^{-1} and 3430 cm^{-1} corresponds to N-H stretching vibrations in the pyrrole skeletal ring, of PS, PC and PT respectively. The peaks near 2928 cm^{-1} and 2852 cm^{-1} corresponds to the C-H stretching vibration of the methylene group. The intensity of these peaks was so weak which indicated that surfactants had been completely removed from these polymers. [Zhang et al., 2006].

The most pronounced change after the deprotonation was the reduction of absorption above 1800 cm^{-1}. An additional peak at about 1748 cm^{-1} and 1710 $cm^{-1}$was observed for all samples, indicating that PPY got slightly over oxidized during the growth process. The oxygen might entered the PPY structure during the polymerization process itself as a consequence of the water present in polymerization solution, as well as by reaction of the prepared polymer with atmospheric oxygen. These peaks were assigned to C=O bond carbonyl groups, formed through oxidative polymerization. These peaks were missing after enzyme immobilization indicating the adsorption of enzyme on the surface of polypyrrole supports. This also underlines the fact that polypyrrole can be directly used as support for enzyme immobilization without any previous functionalization [Sandu et al., 2011].

The peak around 1028 cm^{-1} indicates N-H ring out of plane bending which get shifted to 1024cm^{-1} for PC and 1020 cm^{-1} for PT. The C-H ring out of plane bending around 592 cm^{-1} was seen in all samples.

For all samples there observed a shift to lower wave number after enzyme had been immobilized. For all three samples, the characteristic peaks with respect to enzymes were also obtained. As per the literature review, the main peaks around 1656 cm^{-1}, 1646 cm^{-1}, 1617 cm^{-1}, 1596 cm^{-1}, 1542 cm^{-1} and 1397 cm^{-1}were the characteristic peaks confirming the presence of diastase alpha amylase.

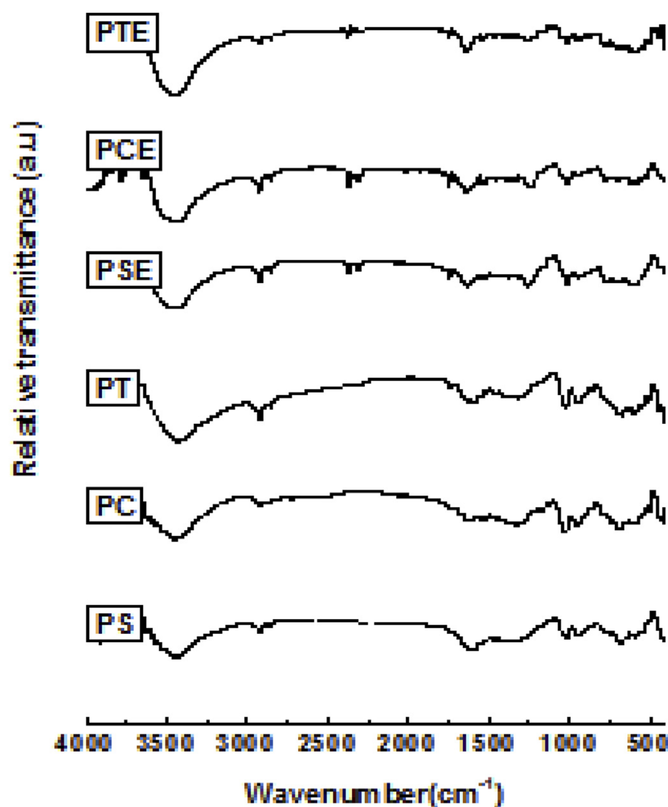


Fig. 1a. IR spectrum of polypyrrole prepared in presence of surfactants.

Table 1
Surface area of PPY prepared in presence of surfactants and their enzyme immobilized forms (PSE, PCE & PTE).

Polymers	Surface area (m^2/g)
PS	9.4
PC	11.9
PT	26
PSE	5.96
PCE	9.07
PTE	23.3

3.2. Surface area analysis

The BET surface area analysis of the polymers before and after immobilization was carried out and the results are depicted in the Table 1. The yield of polymer formed and size of particles were affected by the type of surfactant as the surfactant was adsorbed physically to the growing polymer chain. Surfactants prevented the gross aggregation of particles, thereby influenced the rate of polymerization and decreased the particle size. As relatively low temperature was employed during reaction condition, the mobility of surfactant was restricted, leading to the decrease in the inner volume of micelles that encapsulated the monomer and the oxidant (Reung-U-Rai, A, 2008). Reduced micelle volume resulted in reduced particle size. Structural variation in the surfactants resulted in the formation of polypyrroles with varying particle size. The enzyme immobilized samples of all polymers showed corresponding decrease in surface area because of adsorption of diastase alpha amylase on the surface of the polymers. Protein loading was found to be proportional to the surface area of polymers. Surface area and protein loading of polypyrrole prepared in presence of Tween -80 was found to be more compared to others which might be due to

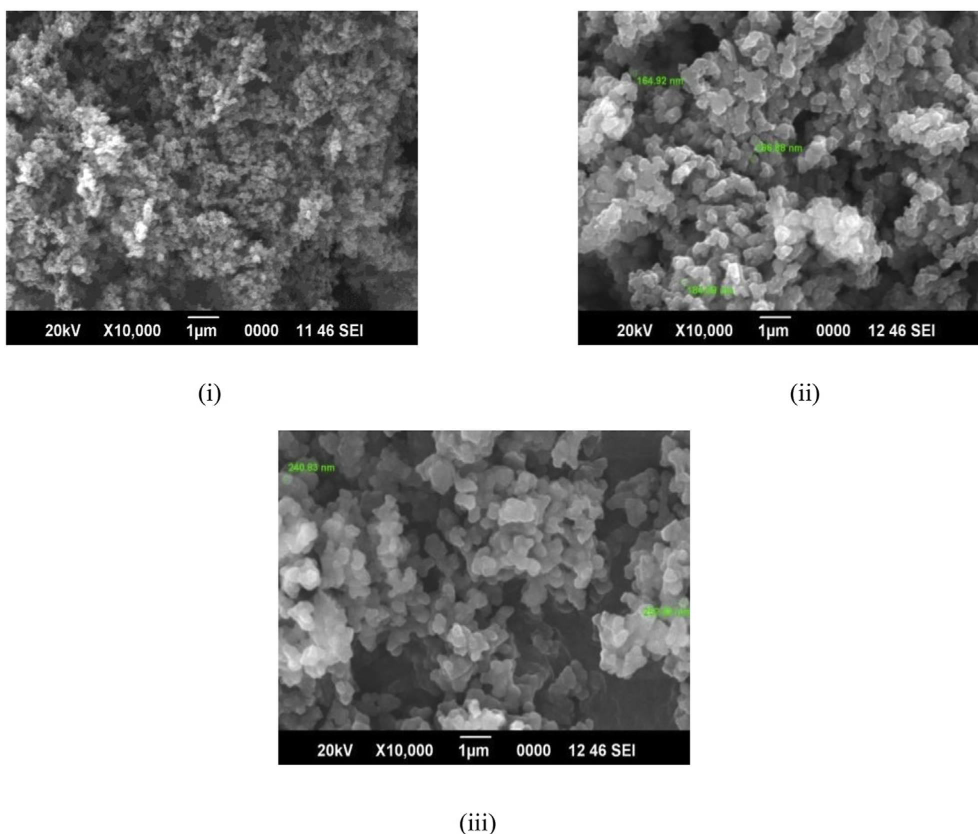


Fig. 1b. SEM images of (i) PS (ii) PC (iii) PT.

lower particle size of formed polymers which inturn was influenced by structure of the surfactant.

3.3. Scanning electron microscopy

The SEM images showed that the polymer obtained has spherical structures. The surfactants played a major role on the surface morphology of products. The total surface area was increased upon the removal of the surfactant template. In the SEM images agglomeration of particles was also observed. This might be due to the close interactions between the polymer chains. The SEM images obtained are shown in Fig. 1b.

3.4. Immobilization of alpha-amylase on polypyrrole prepared in presence of different surfactants.

3.4.1. Influence of pH during immobilization of diastase alpha amylase on polymer supports

The immobilization of alpha-amylase in different pH medium was carried out so as to optimize pH for binding. The results obtained are shown in the Fig. 2a.

The polypyrrole has an isoelectric point around 7 (Perruchot, C.et al., 2008).The amylase isoelectric point is around 4.6. This promoted adsorption of diastase alpha amylase easily on to polypyrrole. Adsorption nature was investigated in different pH's of buffer solutions that are in the range 4-8 because amylase becomes unstable above pH 9.0. Electrostatic interactions occured when enzyme and support were oppositely charged and repulsion occured when both are likely charged and that sometimes resulted in lower adsorption. At lower and higher pH, hydrophobic interactions dominated over electrostatic ones and resulted in high adsorption rate irrespective of pH effect. The immobilized enzyme activity was highest in the range of pH 5-6. The decrease in activity was observed above and below this pH range. This

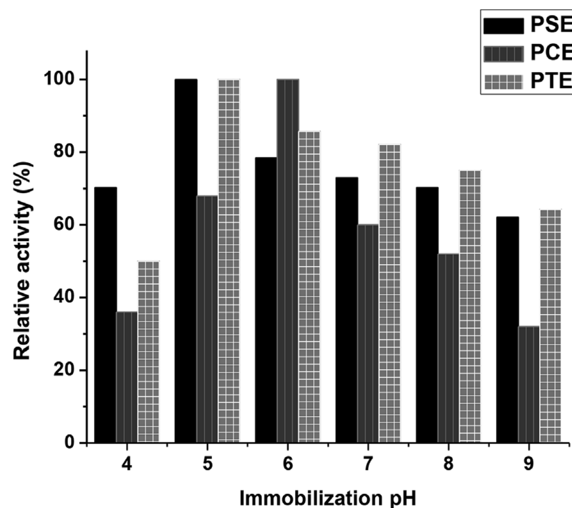


Fig. 2a. Effect of pH of immobilization medium on the relative activity of immobilized alpha - amylase.

can be attributed to lower loadings which occurred as a result of change in conformation of tertiary structure due to unfavourable charge distribution of amino acid residues at lower and higher pH.

Since the free enzyme used in this experiment was stable in the narrow pH range 4.5-5.5, the possible denaturation of enzyme in alkaline region was also expected.

From the experimental data obtained, it was found that pH 5 was best for the immobilization of alpha - amylase on PS and PT whereas, for PC the best retention of enzyme activity was observed at pH6. At pH 5 and 6, since polypyrrole is positively charged and amylase negatively charged, there emerged significant electrostatic interaction between

supports and the enzyme.

Furthermore, all of the polymers have conjugated rings for hydrophobic interactions. At pH 4, PPY adsorbents have overall net positive charge and lysine and arginine amino acid residues on the protein surface of amylase have slight positive charge as pH 4 is close to its isoelectric point. Hence PPY adsorbent should repel diastase alpha amylase enzyme. However, adsorption of proteins was still observed at pH 4 because hydrophobic interactions appeared to dominate over electrostatic interaction at pH 4. The small adsorption difference due to pH originates from ionic effects, due to secondary amino groups of the pyrrole rings.

At pH 7 PPY has no charge whereas amylase is negatively charged. Hence the observed adsorption might have occurred as a result of hydrophobic interaction. When the immobilization is carried out at higher pH, the same amount of enzyme get immobilized, however the activity observed was lower than that attained at pH 5 and 6. This is because at higher pH PPY adsorbents have overall negative charge and diastase alpha amylase is also having net negative charge which results in electrostatic repulsion. Thus overall activity of the enzyme is very much dependent on the strength of the electrostatic interaction between enzyme and the support [Wang, 2004].But the decrease in activity may also be due to diffusional limitations of the substrate towards the active site of the enzyme. However, immobilized enzyme was found to be more resistant towards pH changes compared to native enzyme.

3.4.2. Effect of contact time on the activity of α-amylase

The contact time needed for enzyme to get adsorbed efficiently on PPY adsorbents is shown in Fig. 2b.

For PS the maximum enzyme adsorption capacity was attained within 180-240 min contact time whereas in the case of PC and PT, the contact time was 240 min and 120 min respectively. The decrease in activity after this optimum time might be due to the fact that, as the first adsorption occurred, the surface of the support get saturated with the enzyme and the remaining enzymes in solution had to bind with the support surface via second adsorption site which needed more energy. This could have weakened the adsorption efficiency. The enzyme immobilized polymer samples will be designated as PSE, PCE and PTE respectively.

3.4.3. Effect of initial protein concentration on protein loading on to polymeric supports

The amount of protein bound to PPY adsorbents were analyzed based on the optimized conditions obtained above; it is shown in Fig. 2c.

When the effect of initial protein amount on protein loading was monitored, it was found that the enzyme adsorption profoundly increased with increase in the enzyme concentration [Usman et al., 2013], but after a particular concentration, no further increase in adsorption occurred; instead reached at a saturation point. This could be due to partial dissociation of enzyme subunits as a result of immobilization procedure.

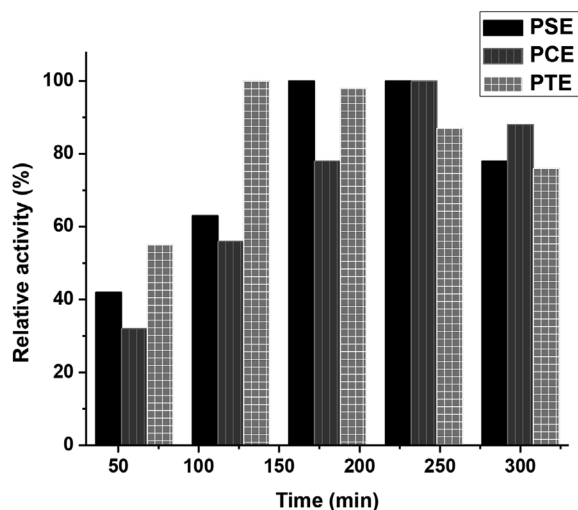


Fig. 2b. Effect of contact time on immobilized enzyme activity.

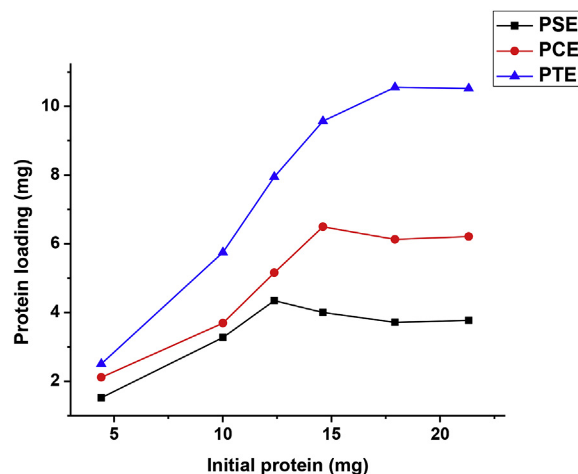


Fig. 2c. Effect of initial protein amount on protein loading.

3.4.4. Effect of initial protein concentration on immobilization yield and activity of loaded protein

Immobilization yield obtained for all adsorbents at various enzyme concentrations are shown in Fig. 2d.

From the graph, it is clear that enzyme loading increased as concentration was increased which then reaches a saturation point and then decreased or remains constant which might be due to diffusional limitation of substrate towards the active site of the enzyme.

For PS optimum immobilization yield was 34% when 12.8mg of initial protein was added. Protein load was also the maximum at this concentration with immobilized enzyme activity 26.52 EU.

For PT even if maximum loading of 65.5% was obtained when initial protein was 14.6 mg and protein load 9.6 mg, the optimum immobilized activity 12.2EU was obtained at the initial protein concentration of 17.9 mg with protein load of 10.6 mg. Similar is the case with PC.

The trend of immobilized enzyme activity when initial protein

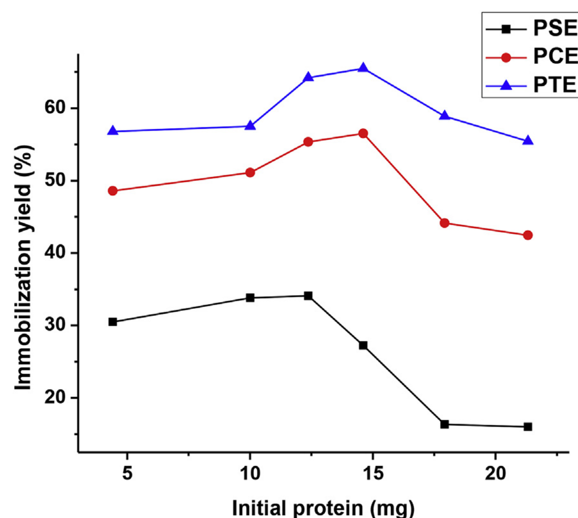


Fig. 2d. Effect of initial protein concentration on immobilization yield of enzyme.

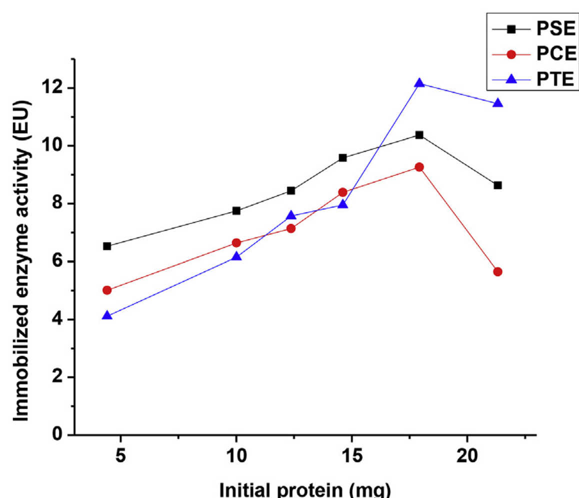


Fig. 2e. Effect of initial protein concentration on immobilized enzyme activity.

amount was varied for all adsorbents is shown in Fig. 2e.

The immobilization yield, activity yield and immobilization efficiency were also evaluated. The results are tabulated in Table 2.

Because of comparatively smaller particle size and high surface area, protein loading was the maximum for PT with respect to PS and PC. Correspondingly, activity yield was also found higher for PT. But the immobilization efficiency was highest for PS compared to PC and PT. Thus results indicate that loading efficiency was affected by the strength of interaction between the enzyme and the support which in-turn depends upon the protein loading, pH of the medium, time of immobilization, temperature etc.[Demirkan et al., 2011].

3.4.5. Effect of pH on enzyme activity

An enzyme's apparent response to pH may change when it is in a heterogeneous environment associated with polymer matrices. Fig. 3a shows the effect of different pH on enzyme activity.

Free and immobilized diastase alpha amylase exhibit similar activity in the range from pH 4.5 to 5.5. In the case of PSE optimum activity was observed at pH 5 whereas, optimum activity in the case of PCE and PTE was at pH 6. Above pH 6.0, immobilized diastase alpha amylase showed better performance than free diastase alpha amylase.

At pH 4 and 8 a decrease in the enzymatic activity was observed for both immobilized and the free enzyme; however, at pH 8 the residual activity of the immobilized enzyme in most samples was significantly higher than that of the unmodified form.

A greater bulk pH is required for providing an optimum pH in the microenvironment of the enzyme and hence a shift to higher value was encountered. Thus, the immobilization process provides structural stability, preventing an irreversible unfolding of the enzyme protein.

The enzyme was inactivated at lower pH values (pH < 5) [Vinu et al., 2004]. The curve profile for all immobilized enzymes became much broader when compared to that of free enzyme. The results are shown in Table 3.

3.4.6. Effect of temperature on the activity

The inactivation rate of an enzyme increased with temperature.

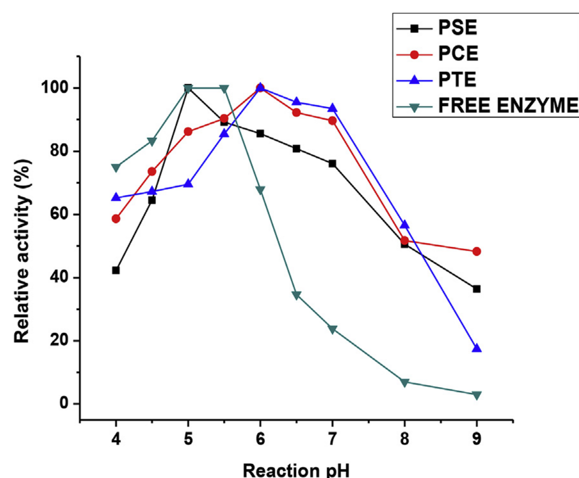


Fig. 3a. Effect of pH on the activity of free and immobilized α – amylase.

Table 3
Optimum pH for free enzyme and immobilized enzymes.

	Free enzyme	PSE	PCE	PTE
pH	5 & 5.5	5	6	6

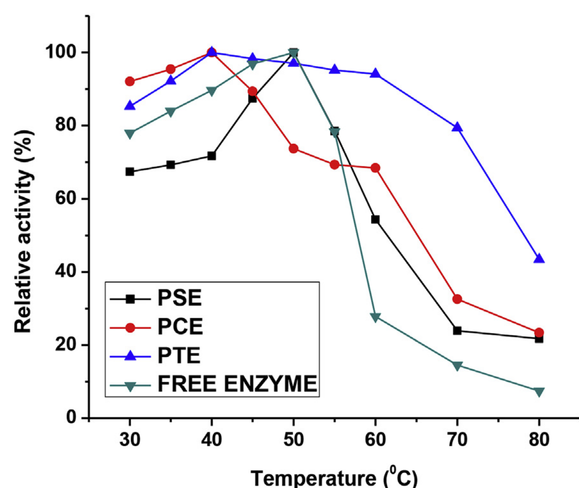


Fig. 3b. Effect of temperature on the activity of free and immobilized α - amylase.

Influence of temperature on activity of free and immobilized diastase alpha amylase is depicted in Fig. 3b.

The 10°C decrease in the optimum temperature combined with thermal stability exhibited by PCE and PTE was an interesting finding of this work. Similar decrease in optimum temperature was reported by Su et al. when they immobilized β-glucosidase on alginate by combining cross-linking with entrapment and, again, cross-linking [Su et al., 2010]. The energy reduction and lower time to cool the reaction mixture represents advantages acquired with the immobilization

Table 2
Immobilization efficiency for alpha - amylase on PPY prepared in presence of surfactants.

Polymer	Initial protein mg	Immobilized protein mg/g	Immobili-zation yield (IY)	Initial Activity EU	Immobilized enzyme activity EU	Activity yield (%) AY	Immobiization efficiency (%) IE = AY/IY
PS	12.8	4.4	34	26.5	8.5	32	92
PC	17.9	6.1	44	27	9.3	34	78
PT	17.9	10.6	59	27	12.2	45	76

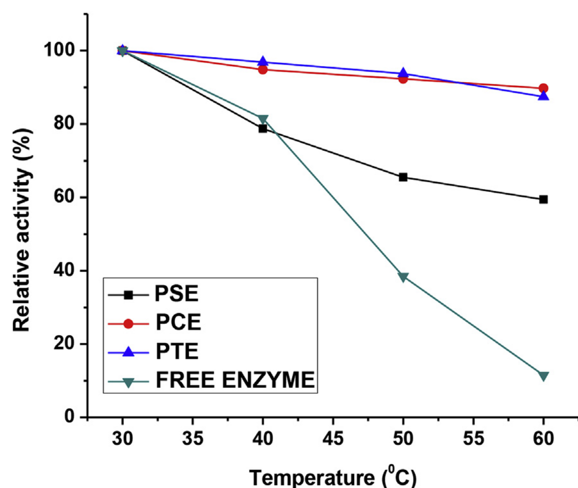


Fig. 4a. Thermal stability of free and immobilized α-amylase.

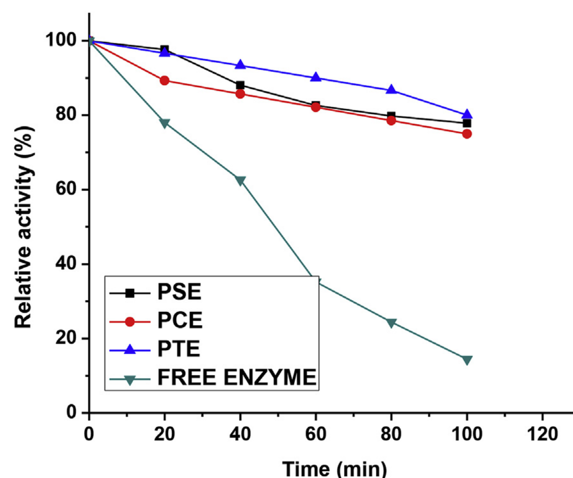


Fig. 4b. Effect of pre-incubation time on the activity of free and immobilized α-amylase.

process.

In the case of PSE there was an increase in optimum temperature by 10°C compared to free enzyme. Since in the bound state, enzymes are less mobile and they resist denaturation of protein [Tanriseven and Doan, 2001]. The decrease in optimum temperatures of PCE and PTE might be due to less activation energy required for starch hydrolysis because of the conformational change that occurred at the enzyme active site after immobilization. But increase in temperature for PSE can be ascribed to the increase in activation energy required for starch hydrolysis as a result of structural changes encountered at the active site after immobilization or due to improper transport of substrate molecules from the bulk to the enzyme active site on account of diffusional resistances to mass transfer [Bahar and Celebi, 1999].

3.4.7. Thermal stability of the free and immobilized enzymes

Thermal stability obtained after immobilization to PPY adsorbents followed the trend as shown in Fig. 4a. After pre-incubation at various temperatures in the range 30–60°C for 1 h with the support in respective buffer solution, 80% of PCE and PTE remained active whereas PSE showed 60% of its initial activity. This improvement in denaturation resistance of the immobilized diastase alpha amylase was probably a consequence of the multipoint attachment acquired in the immobilization process.

The results obtained confirmed the stability of immobilized enzymes, as free enzyme cannot withstand such a prolonged period of thermal treatment [Kikani et al., 2013]. But for industrial application the enzyme should be stable towards temperature fluctuations. The immobilized enzyme showed moderate decrease in activity, which emphasizes that the rate of inactivation was lowered upon immobilization.

As the temperature increases, the stability drops significantly for both free and immobilized amylase. At 40°C, both free and immobilized enzyme retain up to 70-80% of their activity. At 50°C the immobilized enzyme was inactivated at a much lower rate than the free enzyme. The free enzymes lost almost 90% of their activity at 60°C after 1 h treatment whereas immobilized amylase lost only 20-40% of its initial activity. Fig. 4b shows the effect of pre –incubation time on the activity of each immobilized enzyme.

About 70-80% of immobilized enzymes maintained their activity when subjected to 120 min of pre-incubation at their respective optimum temperature whereas, free enzyme could retain only 10% of their initial activity when subjected to same treatment.

These results suggest that the thermal stability of alpha amylase increased considerably as a result of immobilization on to PPY adsorbents and is suitable for long term applications. Such an improvement in thermal stability was observed by Talekar and Chavare when they carried out immobilization of alpha amylase on to calcium alginate via entrapment method [Talekar and Chavare, 2012]. It is reported that immobilization can help to distribute the thermal energy imposed to the protein at higher temperatures and hence it is less susceptible to temperature induced conformational changes.

3.4.8. Determination of kinetic parameters

The kinetic parameters comprising Michaelis constant K_m and maximum rate V_{max} were calculated from the Lineweaver- Burk plots. The plots are presented in Fig. 5.

The K_m values increased and V_{max} values decreased for PSE, PCE and PTE which is in well accordance with the trends reported by other authors[Nehete et al., 1987; Lee et al., 1980].

This demonstrates that there was an eloquent role played by mass transfer restrictions due to diffusional limitations. Since immobilization process may cause disparity in the usual native conformation and thus changes the property of the active site thereby hindering the active site from binding the substrates effectively [Bayramoglu et al., 2008].The increase in Km value thus showed lower affinity of enzyme for substrates and consecutivey lowered enzyme activity compared to free enzyme.The results are consolidated in Table 4.

3.4.9. Storage stability of immobilized alpha amylase

In the free form, enzyme has very short life time and hence gets easily inactivated with minor fluctuations in its local environment. Immobilization of enzymes enabled long-term storage of the enzyme and thus makes it available for various applications. In the dry form, the immobilized enzymes cannot maintain their stability and activity for long term storage. But, when stored in buffer solution under low temperature of 4°C immobilized enzymes exhibited better activity and could retain its stability.

On the other hand free enzyme even when stored in buffer solution under low temperature conditions lose complete activity within 7 days. Whereas PCE could retain 53% of its initial activity, PTE retained 60% of its initial activity and PSE 40% of its initial activity, all of which were far better than free enzyme as can be seen in the Fig. 6a. This decrease in activity among the immobilized enzymes can be explained as the time-dependent natural loss in enzyme activity which occurs due to conformational swaps in the active site of enzyme on long term storage that is caused by revamps in its micro environment. Similar enhancements in storage stability after immobilization have been reported by other authors[Cakmakci et al., 2013, Kharkrang and Ambasht, 2013].

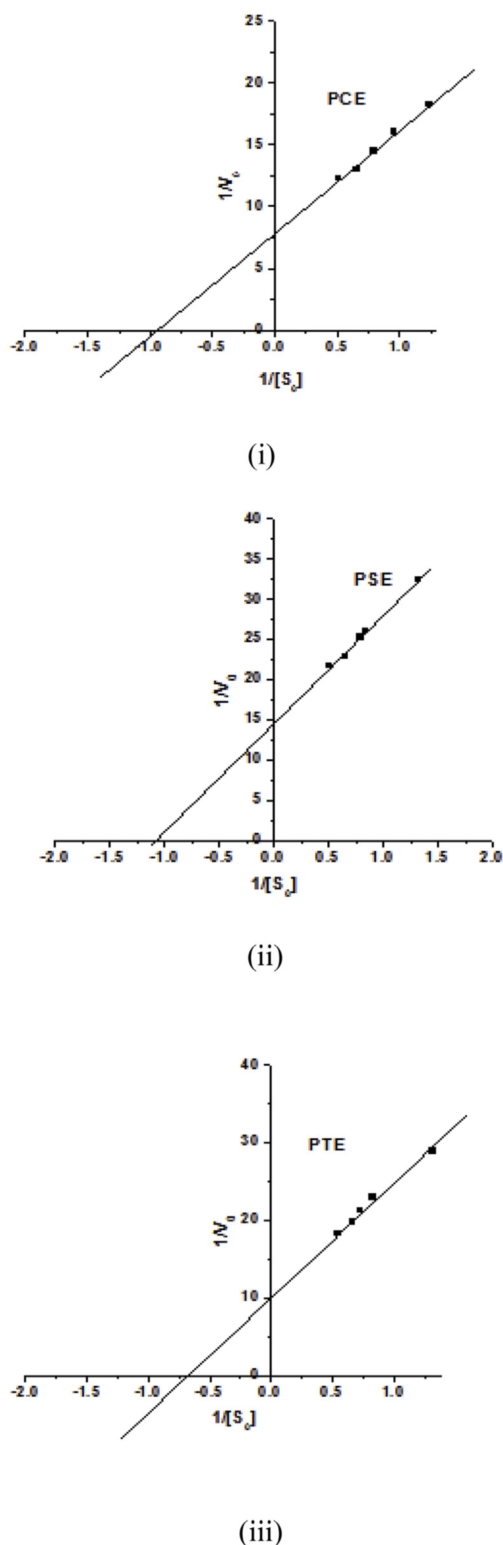


Fig. 5. Lineweaver –Burk plots for (i) PSE (ii) PCE (iii) PTE.

Table 4
Kinetic parameters determined for free and immobilized alpha-amylase.

	Free Enzyme	PSE	PCE	PTE
K_m(mg/ml)	0.50 ± 0.04	0.916 ± 0.01	1.07 ± 0.04	1.49 ± 0.05
V_{max}(mg/ml/min)	7.40 ± 0.05	2.34 ± 0.03	4.41 ± 0.02	3.44 ± 0.02

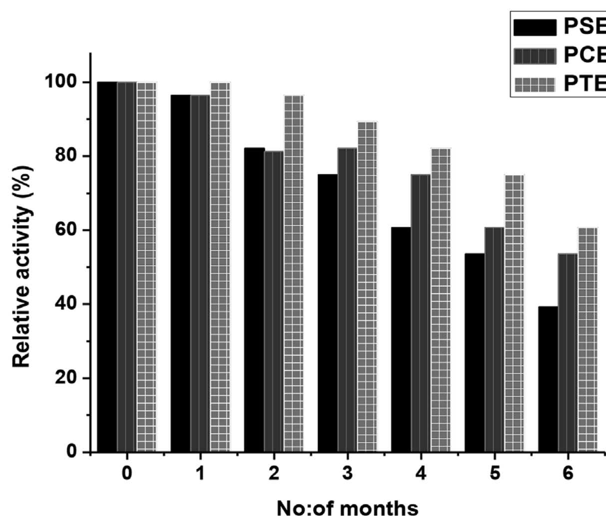


Fig. 6a. Storage stability of immobilized enzymes.

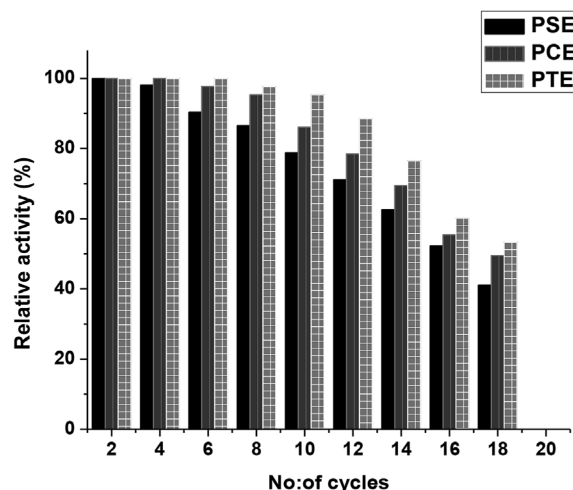


Fig. 6b. Reusability of the immobilized enzymes.

3.5. Reusability

In our systems, reusability was checked for 20 continuous cycles and the results obtained were given in the Fig. 6b. After 15 cycles, PSE and PCE retained 50% of their initial activity whereas; PTE retained almost 60% of its initial activity. It was observed that the immobilized enzyme activity decreased when recycling number was increased.

Jaiswal et al. reported that immobilized alpha-amylase on gelatin was reusable up to seven cycles. Almost 90% activity was retained up to three cycles, but with subsequent runs, there was a decline in the activity of the immobilized enzyme. The activity loss upon reuse could be due to weakening in the strength of bond between the matrix and enzyme on repeated use and hence the enzyme might leach out from the matrix, bringing about amendment in its activity in the subsequent cycles[Jaiswal and Prakash 2011]. Besides, the frequent encountering of the substrate into the same active site might distort it which would retard the catalytic efficiency either partially or entirely [Swarnalatha et al., 2013]. Om Prakash et al. Reported that alpha–amylase immobilized on agarose and agar matrices could retain its activity up to 5 cycles after which there was a subsequent decrease in activity which can be associated to enzyme denaturation and due to physical loss of enzyme from the carrier[Prakash and Jaiswal, 2011].

4. Conclusion

This work describes the immobilization of diastase alpha amylase under very mild condition using simple adsorption method on to polypyrrole polymer prepared using surfactant as templates. Various immobilization parameters have been investigated and optimized parameters were used as basis for interpreting the changes occurred for diastase alpha amylase after immobilization. Immobilization yield, activity yield and Immobilization efficiency were also examined for all polymers. Finally the results were compared for all with the free enzyme before immobilization.

The surfactants played a major role on the surface morphology of products, upon the removal of the surfactant template, the total surface area increased which in turn affected the protein loading of each polypyrroles prepared. Thus by properly tailoring the surface morphology of polypyrrole, we could utilize it for many biochemical and biosensor applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101164.

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